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Inhibition of N-glycosylation induces tyrosine sulphation of hybridoma immunoglobulin G

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Immunoglobulin G2a (IgG2a) secreted by the hybridoma line M 31 was found to contain covalently linked sulphate. The sulphate was bound to the heavy chain which existed in several isoelectric variants. All variants were sulphated, the more acidic ones being more highly sulphated. Within the heavy chain the sulphate was not linked to tyrosine, threonine or serine residues, but appeared to be bound to N-linked oligosaccharides located in the Fab-portion. In contrast, the N-linked oligosaccharides in the Fc-portion were unsulphated. Surprisingly, the unglycosylated IgG secreted in the presence of tunicamycin, an inhibitor of N-glycosylation, was not unsulphated, but contained four times as much sulphate on the heavy chain as control IgG. All isoelectric variants of the non-glycosylated heavy chain contained sulphate. This sulphate was localized in the Fc-portion and was largely bound to tyrosine residues. These results show that, upon inhibition of N-glycosylation, the IgG is not simply secreted in non-glycosylated form, but has undergone a different post-translational modification, tyrosine sulphation. We discuss the possibility that tyrosine sulphate residues functionally compensate for the absence of N-linked (sulphated) oligosaccharides in IgG. One common function for these two protein modifications could be to serve as signals for the secretion of IgG.

Key words: hybridoma IgG2a/N-glycosylation/protein secretion/tunicamycin/tyrosine sulphate

Introduction

The sulphation of proteins on tyrosine residues is a widespread post-translational modification (Huttner, 1982). Tyrosine sulphation of proteins has been found in all vertebrate species studied. Within a given species this modification has been observed in most, if not all, tissues. Within a given tissue, several tyrosine-sulphated proteins have been detected. In non-vertebrate species, tyrosine sulphation has been found in insects, e.g., *Drosophila melanogaster* (Baeuerle and Huttner, in preparation), mollusks (Baeuerle and Huttner, unpublished data), and green algae, e.g., *Volvox carteri* (Huttner, Wenzl and Sumper, unpublished data), indicating that tyrosine sulphation occurred early in evolution.

Among the tyrosine-sulphated proteins that have so far been studied are several with known biological roles. These include proteins involved in blood clotting such as fibrinogen (Bettelheim, 1954) and hirudin (Petersen *et al.*, 1976), proteins of the complement system such as C4 (Karp, 1983), yolk proteins such as the vitellogenins of *D. melanogaster* (Baeuerle and Huttner, in preparation), extracellular matrix proteins such as fibronectin (Liu and Lipmann, 1984), and various peptide hormones and neuropeptides including their precursors such as gastrin (Gregory *et al.*, 1964), cholecystokinin (Mutt and Jorpes, 1968) and leu-enkephalin (Unsworth *et al.*, 1982). In addition, many tyrosine-sulphated proteins with unknown biological roles have been detected. Some of these latter proteins (listed in Huttner, 1984) have been characterized with regard to their cellular and subcellular localization (e.g., Rosa *et al.*, in preparation). These studies and a comparative analysis of the tyrosine-sulphated proteins with known biological roles revealed one common denominator: all tyrosine-sulphated proteins known so far are secretory proteins.

The functional diversity of the known tyrosine-sulphated proteins raises the possibility that the primary role of tyrosine sulphation may not lie in its effects on the functional properties of these proteins after their secretion. Rather, tyrosine sulphation may somehow affect the process by which proteins are secreted. A functional role in protein secretion has also been considered for N-linked oligosaccharides of certain secretory proteins. However, in the case of IgG, inhibition of N-glycosylation by tunicamycin did not significantly reduce the secretion of IgG (Hickman and Kornfeld, 1978). Using a hybridoma cell line, we now report that the unglycosylated IgG secreted in the presence of tunicamycin, in contrast to the glycosylated IgG secreted under control conditions, is post-translationally modified by sulphation of tyrosine residues located in the Fc-portion of the heavy chain. We suggest (i) that tyrosine sulphation, in the case of IgG secretion, functionally compensates for the absence of N-linked oligosaccharides, and (ii) that tyrosine sulphation is one, but not the only, protein modification which serves as a signal for the secretion of certain proteins.

Results

Cultures of a hybridoma cell line were labeled with either [^{3}H]tyrosine or inorganic [^{35}S]sulphate, and secreted IgG2a was purified from the culture medium by protein A affinity chromatography. The IgG was analyzed by SDS-polyacylamide gel electrophoresis under reducing (Figure 1a and b) and non-reducing (Figure 1c and d) conditions, followed by fluorography. IgG contained radioactive sulphate on the heavy chain, as shown by the presence of a sulphate-labeled 55-kd band in reducing gels of the material obtained after protein A affinity chomatography (Figure 1b) and after antigen affinity chromatography (not shown). No sulphate label was detected in the light chain, as determined by prolonged fluorography or by liquid scintillation counting. In addition to the 55-kd heavy chain, a sulphate-labeled band of 60 kd was observed in reducing gels (Figure 1b). Compared with the 55-kd heavy chain, the sulphated 60-kd band incorporated very little [^{3}H]tyrosine (Figure 1a) and was present in small amounts, as determined by Coomassie blue staining (not shown). In non-reducing gels, the sulphate label was exclusively found in the position of the IgG tetramer (Figure

1b). These observations suggested that the sulphated 60-kd band was a minor IgG heavy chain variant (compare Koehler *et al.*, 1978) that was sulphated to a greater extent than the predominant 55-kd heavy chain.

Since the heavy chain is known to be N-glycosylated (for review, see Clamp and Johnson, 1972), the sulphate could have been bound to either carbohydrate residues, tyrosine residues, or both. In an attempt to distinguish between sulphation on carbohydrates and on tyrosine, labeled IgG secreted from control cells was compared with that secreted from cells treated with tunicamycin, an inhibitor of N-glycosylation (for review, see Schwarz and Datema, 1982) (Figure 2 and Table I). The heavy chain of the IgG produced and secreted in the presence of tunicamycin migrated faster during SDS-polyacrylamide gel electrophoresis than that of control IgG, having an apparent mol. wt. of 51 kd (Figure 2b). This 51-kd heavy chain did not contain significant amounts of [^{3}H]galactose (Figure 2d, Table I), [^{3}H]fucose or [^{3}H]mannose (Table I), but was heavily labeled with [^{35}S]sulphate (Figure 2f, Table I). The IgG containing the sulphated 51-kd heavy chain still bound to the antigen affinity resin (not shown).

The sulphated residues of the 55-kd and 60-kd heavy chains of IgG secreted in the absence of tunicamycin, as well as the sulphated residues of the 51-kd heavy chain of IgG secreted in the presence of tunicamycin were analyzed after exhaustive

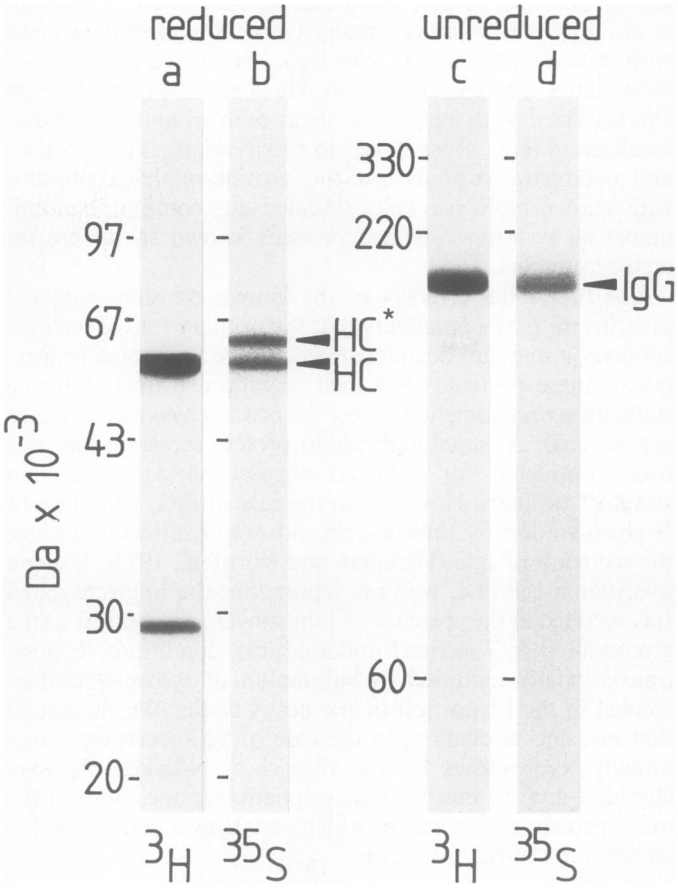

Fig. 1. Sulphation of the IgG heavy chain in hybridoma cells (clone M 31). Fluorograms of 10% **(lanes a** and **b)** and 6% **(lanes c** and **d)** SDS-polyacrylamide gels are shown. IgG labeled with [^{3}H]tyrosine **(lanes a** and **c)** or [^{35}S]sulphate **(lanes b** and **d)** was affinity-purified from the cell culture medium and electrophoresed in the presence **(lanes a** and **b)** and absence **(lanes c** and **d)** of 2-mercaptoethanol. Arrowheads indicate the positions of the heavy chain of 55-kd (HC), of a heavy chain variant of 60-kd (HC*), and of the IgG tetramer (IgG). The positions of mol. wt. standards are indicated next to **lanes a** and **c**.

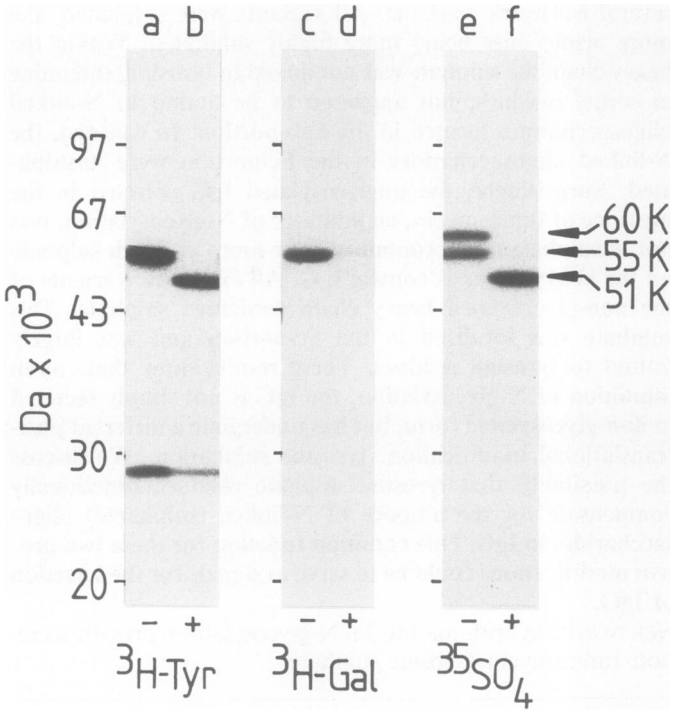

Fig. 2. Sulphation of the IgG heavy chain in the absence and presence of tunicamycin. Fluorograms of 10% reducing SDS-polyacrylamide gels are shown. IgG was labeled in the absence (−) and presence (+) of tunicamycin with [^{3}H]tyrosine **(lanes a** and **b)**, [^{3}H]galactose **(lanes c** and **d)** and [^{35}S]sulphate **(lanes e** and **f)**, and affinity-purified from the cell culture medium. Arrows indicate the positions of the glycosylated 55-kd heavy chain, the glycosylated 60-kd heavy chain variant and the non-glycosylated 51-kd heavy chain. The positions of mol. wt. standards are indicated next to **lane a**.

Table I. Effect of tunicamycin on the glycosylation and sulphation of hybridoma IgG heavy chain

	[^{3}H]Tyrosine	[^{3}H]Fucose	[^{3}H]Galactose	[^{3}H]Mannose	[^{35}S]Sulphate (total)		Tyrosine [^{35}S]sulphate	
Control (55 kd) c.p.m. incorporated	549 525	6212	5333	1016	3216	(100%)	23	(0.7%)
Tunicamycin (51 kd) c.p.m. incorporated	287 680	19	19	20	6439	(100%)	4035	(63%)

Heavy chains labeled with [^{3}H]tyrosine, [^{3}H]fucose, [^{3}H]galactose, [^{3}H]mannose and [^{35}S]sulphate were eluted from single polyacrylamide gel lanes by exhaustive pronase digestion, and radioactivity in the eluates was determined. From the eluate of the [^{35}S]sulphate-labeled heavy chain, only an aliquot was used for counting, and the c.p.m. were extrapolated to give the total [^{35}S]sulphate incorporation. The remainder of this eluate was subjected to tyrosine sulphate analysis, and the radioactivity in the tyrosine sulphate spot was determined. The numbers in brackets in the right column give the percentage of the total sulphate (100%) that was recovered as tyrosine sulphate.

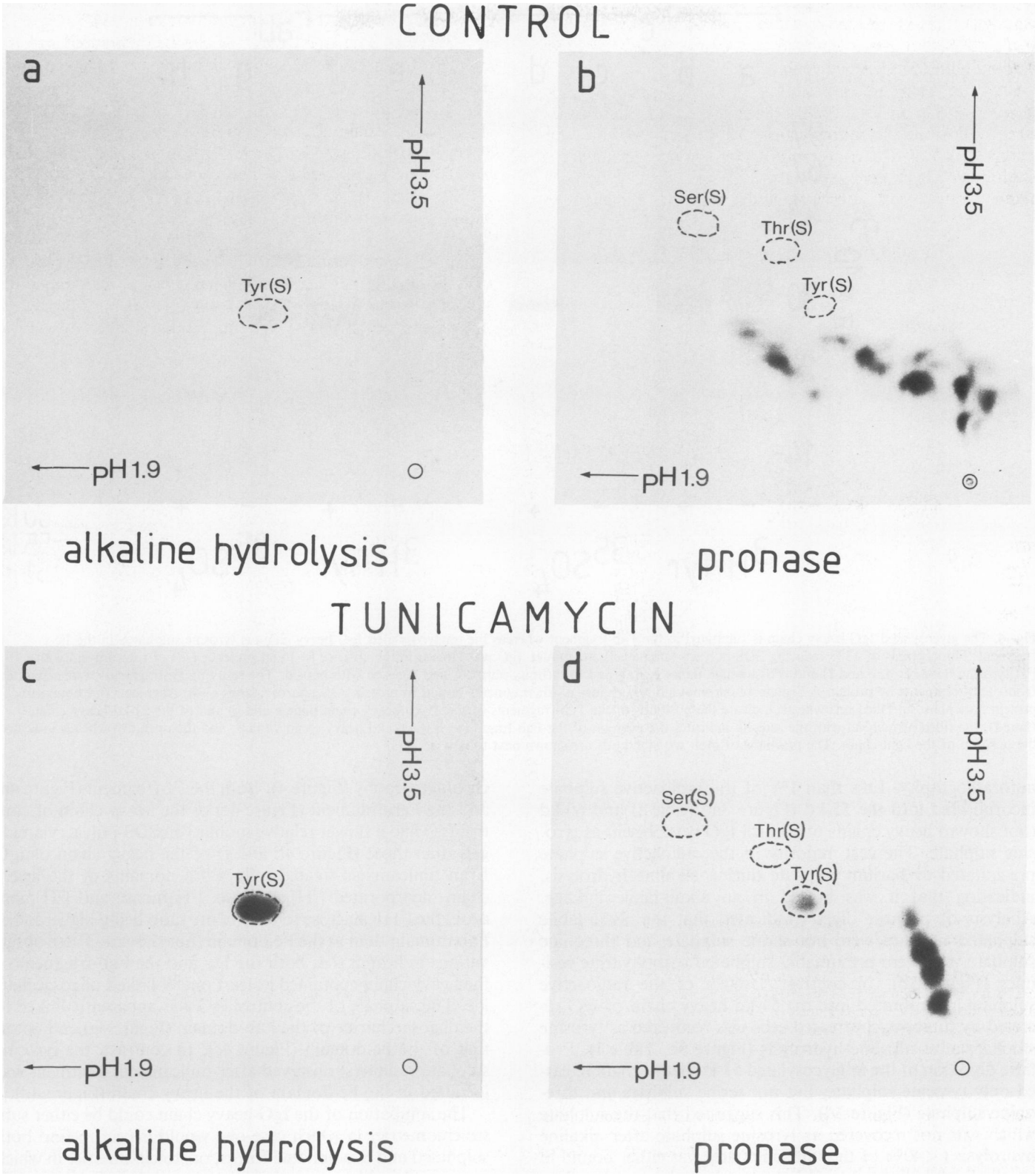

Fig. 3. Inhibition of N-glycosylation by tunicamycin induces tyrosine sulphation of the IgG heavy chain. Autoradiograms of thin-layer cellulose sheets are shown. Alkaline hydrolyzates **(panels a** and **c)** and pronase digests **(panels b** and **d)** of the [^{35}S]sulphate-labeled 55-kd heavy chain of IgG from control cultures **(panels a** and **b)** and of the [^{35}S]sulphate-labeled 51-kd heavy chain of IgG from tunicamycin-treated cultures **(panels c** and **d)** (see Figure 2) were separated by two-dimensional thin-layer electrophoresis as indicated by the arrows. The circle in the lower right corner indicates the origin. Dashed lines show the positions of ninhydrin-stained serin sulphate [Ser(S)], threonine sulphate [Thr(S)] and tyrosine sulphate [Tyr(S)] markers.

alkaline hydrolysis with barium hydroxide (Figure 3a and c). Upon alkaline hydrolysis, tyrosine sulphate is liberated from proteins, while sulphate linked to carbohydrates is largely hydrolyzed and precipitates as barium sulphate (Huttner, 1984; Lee and Huttner, 1983). Tyrosine sulphate was analyzed by two-dimensional thin-layer electrophoresis followed by

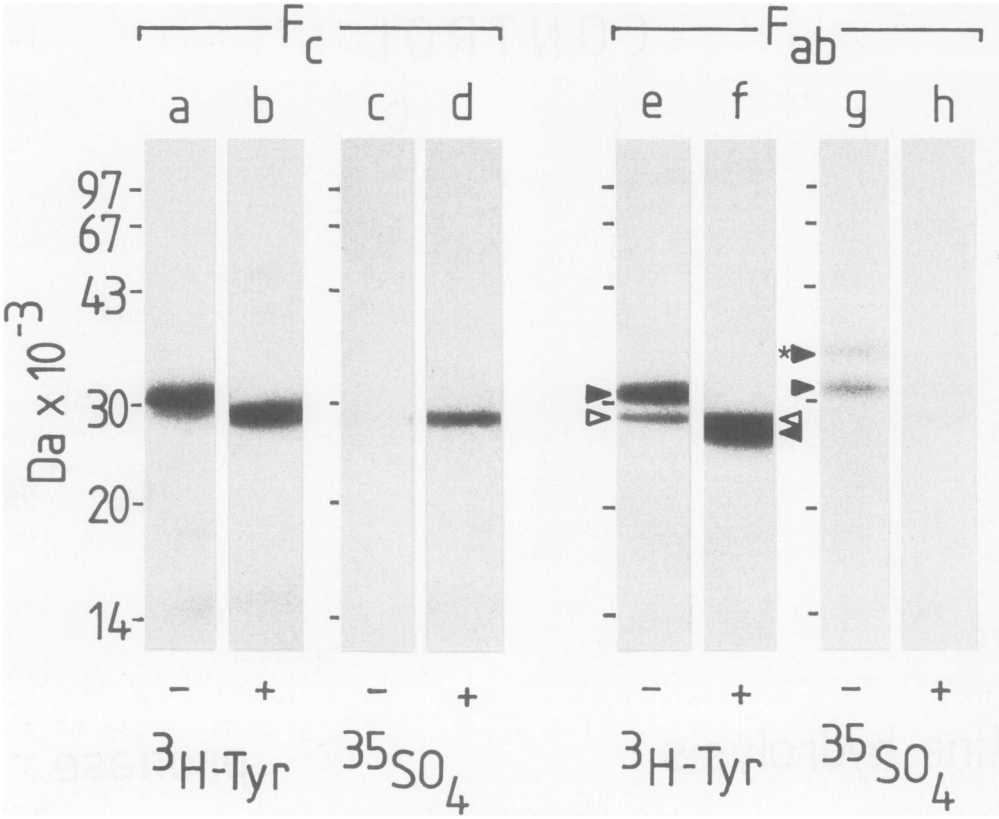

Fig. 4. The glycosylated IgG heavy chain is sulphated in the Fab-fragment whereas the unglycosylated IgG heavy chain is tyrosine-sulphated in the Fc-fragment. Fluorograms of 13% reducing SDS-polyacrylamide gels are shown. IgG was labeled in the absence (−) and presence (+) of tunicamycin with [^{3}H]tyrosine (**lanes a, b, e** and **f**) and [^{35}S]sulphate (**lanes c, d, g** and **h**), affinity-purified, and digested with papain. The resulting Fc-fragments were separated from Fab-fragments by protein A-Sepharose chromatography. **Lanes a–d:** fragments bound to protein A-Sepharose; **lanes e–h:** fragments not bound to protein A-Sepharose. Filled arrowheads indicate the positions of the Fab-fragments of the 55-kd heavy chain (**lane e** and **g**) and of the 51-kd heavy chain (**lane f**), the filled arrowhead with the asterisk indicates the position of the Fab-fragment of the 60-kd heavy chain variant, and the open arrowheads indicate the position of the light chain. The positions of mol. wt. standards are shown next to **lane a**.

autoradiography. Less than 1% of the radioactive sulphate incorporated into the 55-kd (Figure 3a, Table I) and 60-kd (not shown) heavy chains of control IgG was present as tyrosine sulphate. The vast majority of the radioactive sulphate precipitated as barium sulphate during alkaline hydrolysis, indicating that it was bound in an alkali-labile linkage. Analysis of pronase digests indicated that the alkali-labile sulphated residues were not serine sulphate and threonine sulphate, but were presumably sulphated carbohydrate residues (Figure 3b). In contrast, >60% of the radioactive sulphate incorporated into the 51-kd heavy chain of IgG secreted by tunicamycin-treated cells was recovered as tyrosine sulphate after alkaline hydrolysis (Figure 3c, Table I). Pronase digestion of the unglycosylated 51-kd heavy chain released only tyrosine sulphate, but not serine sulphate and threonine sulphate (Figure 3d). This suggested that the sulphate which was not recovered as tyrosine sulphate after alkaline hydrolysis (<40% of the total sulphate) was either bound in an alkali-labile linkage to O-linked carbohydrates other than galactose, fucose and mannose, or bound to tyrosine residues and lost during the alkaline hydrolysis procedure. No ^{35}S was found in the positions of methionine, cysteine and cysteic acid when alkaline hydrolyzates were analyzed by one-dimensional thin-layer electrophoresis (Huttner, 1984) and autoradiography.

IgG secreted by control and tunicamycin-treated cells was digested with papain, and the resulting Fc- and Fab-fragments were separated by protein A-Sepharose affinity chromatography (Figure 4). Both the Fc-fragment (Figure 4a) and the Fab-fragment (Figure 4e) of the heavy chain of control IgG had a slower relative mobility in SDS-polyacrylamide gels than those (Figure 4b and f) of the heavy chain of IgG from tunicamycin-treated cells. Both domains of the heavy chain incorporated [^{3}H]galactose, [^{3}H]fucose and [^{3}H]mannose, the [^{3}H]galactose to [^{3}H]fucose ratio being higher in the Fab-domain than in the Fc-domain (not shown). These observations indicated that both the Fc- and the Fab-fragment of the heavy chain contained at least one N-linked oligosaccharide. The sulphate of the control IgG was apparently linked to the oligosaccharide of the Fab-domain (Figure 4g) and not to that of the Fc-domain (Figure 4c). In contrast, the tyrosine sulphate residue(s) observed after tunicamycin treatment was localized in the Fc-domain of the heavy chain (Figure 4d).

The sulphation of the IgG heavy chain could be either substoichiometric, in which case one would expect to find both sulphated and unsulphated forms, or stoichiometric, in which case one would expect to find only sulphated forms. IgG from control and tunicamycin-treated cells was analyzed under reducing conditions by isoelectric focussing followed by SDS-polyacrylamide gel electrophoresis (Figure 5). Several isoforms of the 55-kd heavy chain were detected (compare Koehler *et al.*, 1978), and their proportions, as determined by [^{3}H]tyrosine labeling (Figure 5a) and Coomassie blue-staining (not shown) were similar to those determined by [^{3}H]fucose labeling (Figure 5e). All isoforms contained radioactive sulphate (Figure 5c). The ratios of [^{35}S]sulphate to [^{3}H]tyrosine

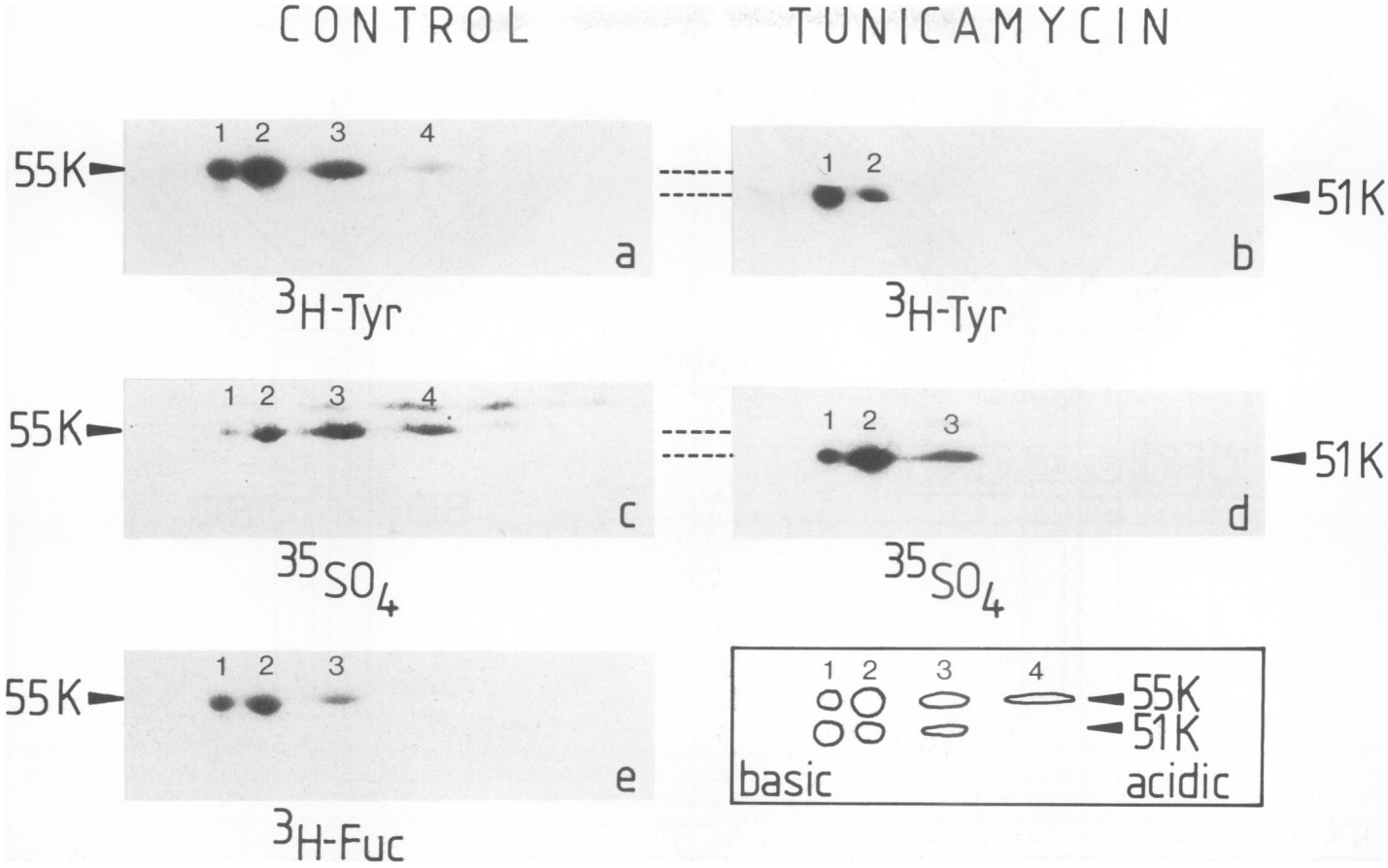

Fig. 5. All isoelectric forms of the heavy chain of IgG secreted by control and tunicamycin-treated cells are sulphated. IgG was labeled with [^{3}H]tyrosine **(panels a** and **b)**, [^{35}S]sulphate **(panels c** and **d)** and [^{3}H]fucose **(panel e)**, affinity-purified from the cell culture medium and separated two-dimensionally, using isoelectric focussing in the first dimension and SDS-polyacrylamide gel electrophoresis in 10% reducing gels in the second dimension, followed by fluorography. The areas of the fluorograms showing the isoforms of the heavy chains are presented. The arrowheads on **panels a, c** and **e** and the upper dotted line indicate the position of the 55-kd heavy chain isoforms of IgG from control cell cultures. The arrowheads on **panels b** and **d** and the lower dotted line indicate the position of the 51-kd heavy chain isoforms from tunicamycin-treated cell cultures. Forms of the 55-kd and 51-kd heavy chains having similar isoelectric points are both designated 1–4, as illustrated in the diagram shown in the lower right panel; left (basic) margin, pH 7.2; right (acidic) margin, pH 6.2.

incorporation into the isoforms were different, the more highly sulphated isoforms being more acidic. Similar observations were made with the isoforms of the non-glycosylated 51-kd heavy chain (Figure 5b), all of which were sulphated (Figure 5d). These results were consistent with the assumption that upon inhibition of N-glycosylation only IgGs containing tyrosine-sulphated heavy chains were secreted.

Discussion

The present results show that IgG secreted in the presence of tunicamycin, an inhibitor of N-glycosylation, is not simply unglycosylated, but has undergone a different post-translational modification, tyrosine sulphation (see model in Figure 6). The most likely explanations of how tunicamycin treatment lead to the tyrosine sulphation of the heavy chain include the following. (i) The absence of the N-linked oligosaccharides from the Fc-portion of the heavy chain may have unmasked tyrosine residues for sulphation by tyrosylprotein sulphotransferase (Lee and Huttner, 1983). (ii) The absence of the N-linked oligosaccharides from the heavy chains may have resulted in a transient accumulation of unsulphated IgG in the Golgi complex. Tyrosine sulphation may then have occurred as a result of the increased concentration of protein substrate.

It seems unlikely that the sulphation of the tyrosine residue(s) in the Fc-domain occurred as an unspecific side reaction resulting from the absence of the physiological substrate for the sulphotransferase responsible for the sulphation of the N-linked oligosaccharide in the Fab-domain. First, in the case of an unspecific side reaction one would not expect the stoichiometric tyrosine sulphation of the heavy chain which apparently occurred since no unsulphated form was detected (Figure 5). Second, sulphation of proteins is catalyzed by tyrosylprotein sulphotransferase, a Golgi enzyme with a high affinity ($K_m < 1$ μM) for appropriate tyrosine residues in proteins, and with certain properties distinct from carbohydrate sulphotransferases (Lee and Huttner, in preparation). While these data support the specific nature of the tyrosine sulphation of the 51-kd IgG heavy chain, they do not exclude the possibility that tyrosylprotein sulphotransferase is also responsible for the sulphation of the N-linked oligosaccharide in the Fab-domain of the 55-kd heavy chain. In fact, it is interesting to note that the tyrosine-specific protein kinase pp60^{src} also phosphorylates phosphatidyl inositol on hydroxyl groups of the inositol ring which is structurally similar to a hexose ring (Sugimoto *et al.*, 1984). Third, the dramatic increase in tyrosine sulphation upon tunicamycin treatment appeared to be specific for IgG. Most, if not all, of the sul-

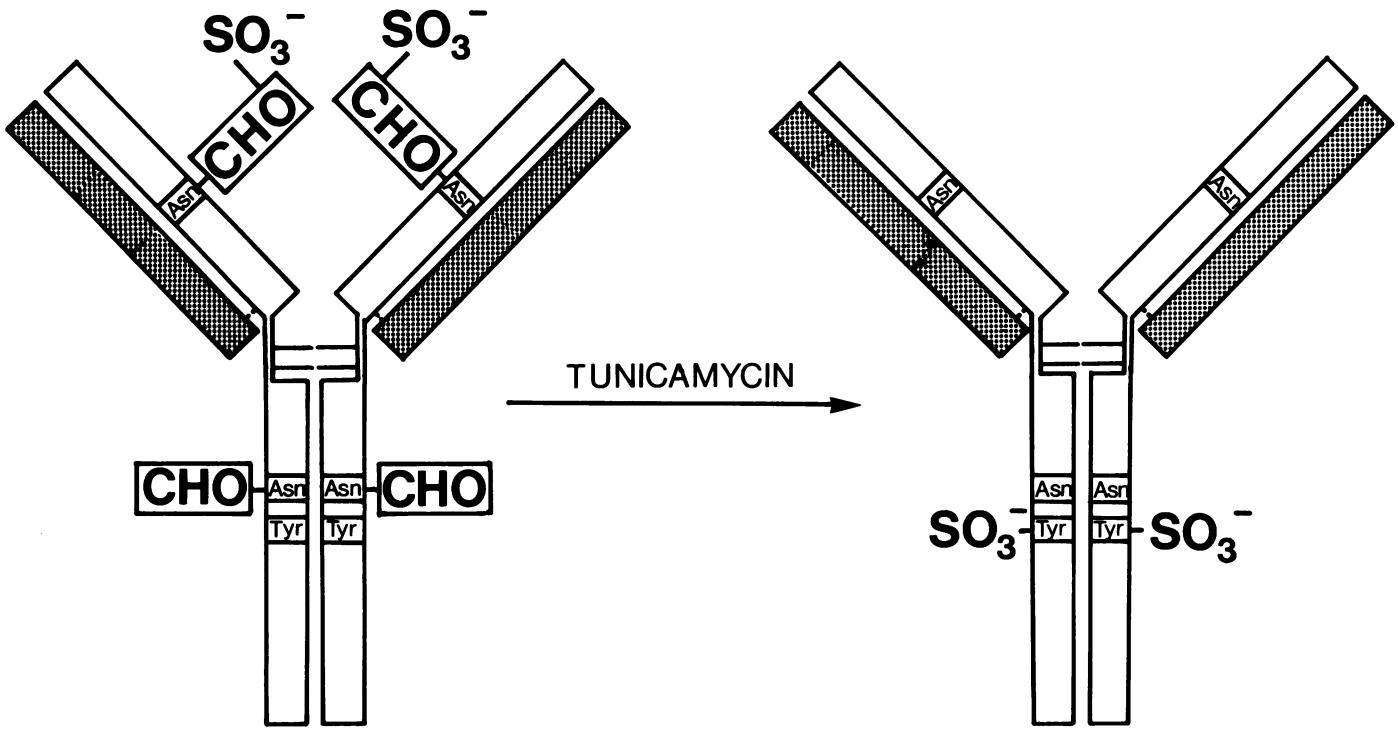

Fig. 6. Diagram illustrating the effects of tunicamycin on the glycosylation and sulphation of IgG2a secreted by the hybridoma cell line M 31. The IgG model on the left shows the known N-linked carbohydrate (Asn-CHO) in the Fc-portion and the presumed N-linked sulphated carbohydrate (Asn-CHO-SO_3^-) in the Fab-portion. After inhibition of N-glycosylation by tunicamycin, a tyrosine residue(s) in the Fc-portion becomes sulphated (Tyr-SO_3^-), as shown in the IgG model on the right. The positions of the ASn-CHO-SO_3^- and the Tyr-SO_3^- residues within the Fab-portion and the Fc-portion, respectively, are arbitrary.

phated proteins found in the cell pellets showed a reduced sulphate incorporation in the presence of tunicamycin (data not shown). This finding also argues against the possibility that the increase in radioactive sulphate incorporation on tyrosine residues of the IgG heavy chain resulted from an increase in the specific activity of 3'-phosphoadenosine 5'-phosphosulphate, the co-substrate for sulphation.

The functional significance of the tyrosine sulphation of the unglycosylated IgG is unclear. It is possible that tyrosine sulphation is somehow required for the secretion of unglycosylated IgG. In the past, the lack of significant inhibition of IgG secretion by tunicamycin has been taken to indicate that N-linked oligosaccharides are not involved in the secretion of IgG (Hickman and Kornfeld, 1978). The present results, showing that in the absence of N-glycosylation the IgG heavy chain undergoes tyrosine sulphation in its Fc-portion, suggest that both modifications may serve similar functions, at least in the present case. For example, N-linked oligosaccharides may have served as one signal for the secretion of IgG, the absence of which was compensated for by the addition of another signal, tyrosine sulphate. A role of tyrosine sulphation in the secretion of certain proteins would be consistent with the observation that all tyrosine-sulphated proteins known today, though functionally distinct, are secretory proteins (Huttner, 1984).

IgG is not the only type of immunoglobulin that can become tyrosine-sulphated. We have recently found that secreted unglycosylated mouse and rat hybridoma IgM are tyrosine-sulphated on the heavy chain (Baeuerle and Huttner, in preparation). It will therefore be of interest to investigate whether other glycoproteins known to be still secreted after inhibition of N-glycosylation are tyrosine-sulphated.

The observation that inhibition of one protein modification induces the occurrence of another, distinct modification is also of interest in a more general context. Secretory proteins are known to undergo several different post-translational modifications. However, no simple common denominator has been observed to explain why some, but not all, secretory proteins are glycosylated, some sulphated, and some phosphorylated. These findings may appear less puzzling if one considers the possibility that distinct modifications may be able to functionally substitute for one another.

Materials and methods

Isotopes

L-[3,5-^{3}H]tyrosine (50 Ci/mmol) and carrier-free [^{35}S]sulphate were purchased from Amersham. L-[5,6-^{3}H]fucose (56 Ci/mmol), D-[2-^{3}H(N)]mannose (24 Ci/mmol) and D-[6-^{3}H(N)]galactose (29 Ci/mmol) were obtained from New England Nuclear.

Cell culture and labeling

The hybridoma cells used were a kind gift of Dr L.J.DeGennaro and were derived from the clone designated M 31 [obtained by fusion of the myeloma cell line Sp2/O-Ag 14 with spleen cells of a mouse immunized with purified synapsin I (DeCamilli *et al.*, 1983) producing IgG directed against synapsin I (L.J.DeGennaro, unpublished results)]. Cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 15% fetal calf serum. For labeling, cells were suspended at a density of 2.5 x 10^6 cells/ml and incubated in sulphate-free DMEM supplemented with 15% dialyzed fetal calf serum, in the absence and presence of 1.5 μg/ml of tunicamycin (Boehringer). After 75 min, either 50 μCi/ml [^{3}H]tyrosine, 5.6 μCi/ml [^{3}H]fucose, 5.6 μCi/ml [^{3}H]mannose, 14 μCi/ml [^{3}H]galactose or 0.5 mCi/ml [^{35}S]sulphate were added to the cell cultures. After 18 h of labeling, each cell suspension was centrifuged at 150 *g* to separate cells and medium.

Affinity purification of IgG

The 150 *g* culture supernatant was further centrifuged at 150 000 *g* for 60 min. The resultant supernatant, referred to as culture medium, was mixed at 4°C with 0.1 volume of 10-fold concentrated buffer A (0.15 M NaCl, 1% (w/v) NP-40, 0.005 M EDTA and 0.05 M Tris-HCl, pH 8.5) and incubated batch-wise for 2 h with protein A-Sepharose (Sigma) pre-equilibrated with

buffer A. The protein A-Sepharose beads were then extensively washed with buffer A, and once with 20 mM sodium phosphate buffer, pH 8.5. IgG was eluted from the protein A-Sepharose with a solution containing 0.15 M NaCl and 0.1 M acetic acid, pH 3.5, and the eluate was neutralized by addition of 0.5 volumes of 0.5 M Tris-HCl, pH 8.5. This material is referred to as affinity-purified IgG. The material was then diluted with four volumes of acetone, and the precipitated IgG was collected by centrifugation and dissolved by boiling in Laemmli sample buffer (Laemmli, 1970) in the absence or presence of 3.3% 2-mercaptoethanol. In some experiments, affinity-purified IgG was incubated batch-wise for 2 h with synapsin I-Sepharose 4B pre-equilibrated in buffer A without NP-40. The synapsin I-Sepharose beads were then washed extensively with buffer A containing 0.3 M NaCl. IgG was eluted from the synapsin I-Sepharose with Laemmli sample buffer containing 2-mercaptoethanol and boiled.

Papain digestion of IgG

Affinity-purified IgG (5 – 50 μg) labeled with [^{3}H]tyrosine or [^{35}S]sulphate was mixed with 650 μg bovine hemoglobin (Sigma, type II). Cysteine was added to a final concentration of 10 mM. After addition of 5 μg papain (Worthington), samples were incubated in a final volume of 1.25 ml for 8 h at 37°C. The reaction was stopped by the addition of 0.2 mM phenylmethylsulphonyl fluoride (PMSF) (Serva), 10 μM leupeptin (Sigma) and 10 mM 4-hydroxymercurybenzoic acid (Calbiochem). Samples were then incubated for 1 h with protein A-Sepharose at room temperature. The protein A-Sepharose was extensively washed with buffer A containing PMSF, leupeptin and 4-hydroxymercurybenzoic acid. To the protein A-Sepharose supernatants, four volumes of acetone were added. The samples were kept overnight at – 20°C and the precipitated protein was collected by centrifugation. The protein pellets and the washed protein A-Sepharose pellets were mixed with Laemmli sample buffer without 2-mercaptoethanol and boiled for 5 min. Protease inhibitors were added in concentrations as described above. The samples were then reduced by boiling in the presence of 3.3% 2-mercaptoethanol, and subjected to SDS-polyacrylamide gel electrophoresis on 13% gels.

Polyacrylamide gel electrophoresis

One-dimensional SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Two-dimensional gel electrophoresis was performed according to O'Farrell (1975) using 3.5% (v/v) ampholytes pH 3.5 – 10 (LKB) and 3.5% (v/v) ampholytes pH 7 – 9 (LKB) in the isoelectric focussing dimension and 10% reducing SDS-polyacrylamide gels for the second dimension. Gels were fixed, stained, destained, and fluorographed as described (Lee and Huttner, 1983). Radioactivity in gel bands was determined after extensive digestion of the protein in the gel with pronase (Huttner, 1984), which was found to result in > 90% elution of the label, followed by liquid scintillation counting in Aqualuma (Baker).

Tyrosine sulphate analysis of IgG heavy chains

[^{35}S]Sulphate-labeled heavy chains from IgG of control and tunicamycin-treated cell cultures were cut from the gels, eluted by pronase digestion, hydrolyzed in 0.2 M barium hydroxide, and the resulting hydrolysates were separated by two-dimensional thin-layer electrophoresis as described in detail by Huttner (1984). Pronase digestion of [^{35}S]sulphate labeled heavy chains without subsequent alkaline hydrolysis (Huttner, 1984) was performed for 30 h at 37°C with a total of 100 μg pronase (Boehringer) in 2 ml per single gel piece. Samples were analyzed by two-dimensional thin-layer electrophoresis. Added sulphated amino acid standards were visualized by ninhydrin-staining. Thin-layer sheets were subjected to autoradiography. Radioactivity in ninhydrin-stained spots was determined by liquid scintillation counting in Aqualuma.

Determination of the IgG subclass

The subclass of the M 31 IgG was determined by the Ouchterlony double diffusion method in the presence of 2.5% polyethylene glycol 4000, using goat and rabbit IgGs specific for the various subclasses of mouse IgG.

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